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(54) Title: ANGIOTENSIN IV AND ANALOGS AS REGULATORS OF FIBRINOLYSIS

(57) Abstract

Angiotensin IV (VAL-TYR-ILE-HIS-PRO-PHE), a degradation product of angiotensin II previously thought to be inactive, interacts directly with endothelial cells to induce expression of PAI-1 and thereby to inhibit clot lysis attributable to endogenous t-PA. Moreover, angiotensin IV does not effect substantial physiological changes (vasoconstriction, increased blood pressure, etc.) characteristic of angiotensin II. Fibrinolysis is promoted by reducing the amount or the effect of angiotensin IV. Fibrinolysis is inhibited by providing enhanced angiotensin IV. Methods of screening candidates for antagonizing angiotensin IV are also disclosed.

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ANGIOTENSIN IV AND ANALOGS AS REGULATORS OF FIBRINOLYSIS

Cross-reference to Related Application

This is a continuation-in-part of the following co-pending U. S. patent applications: a) Serial No. 08/113,292, filed by Douglas E. Vaughan on August 27, 1993; b) USSN 07/906,396 filed June 24, 1992, by Joseph Harding and John W. Wright; and c) PCT/US93/06038 (U.S.S.N. 08/360,874) filed June 24, 1993 by Joseph Harding and John W. Wright, and published as WO 94/00492. Each of the above applications is hereby incorporated by reference.

Statement as to Federally Sponsored Research

This invention was made at least in part using funding from the United States Government, and the Government has certain rights in the invention.

Background of the Invention

This invention relates to the general field of controlling fibrinolysis.

It is well accepted that most mammals, including humans, have mechanisms preventing blood loss when a vessel is severed or ruptured. Specifically, a complex cascade of events culminates in the formation of a blood clot plugging the opening in the vessel in short order after the opening occurs. Once formed, the clot may be

invaded by fibroblasts and eventually be organized into fibrous tissue that will permanently close the opening in the vessel. Alternatively, the clot can dissolve.

When a clot is formed, a large amount of a euglobulin plasma protein known as plasminogen is incorporated in the clot along with other plasma proteins. Plasminogen is activated to dissolve clots by plasminogen activators which convert plasminogen to plasmin, a proteolytic enzyme that digests fibrin threads and other substances in the surrounding blood, causing lysis of the clot. This process is termed fibrinolysis. A particularly important plasminogen activator, known as tissue plasminogen activator (t-PA), has been well studied as therapeutic to treat acute clotting such as occurs with a myocardial infarction.

The balance between clotting and lysis is affected by plasminogen activator inhibitors, particularly an inhibitor known as PAI-1. Expression of PAI-1 involves the renin-angiotensin system. Specifically, angiotensin II, which is formed by the sequential enzymatic cleavage of angiotensinogen, ultimately results in expression of PAI-1. Olsen et al., *Proc. Nat'l. Acad. Sci. USA* (1991) 88:1928-1932. Angiotensin II is also a potent vasoconstrictor. An angiotensin II receptor is known, and blood pressure control therapeutics based on inhibiting formation of angiotensin II (e.g., ACE

inhibitors) and based on antagonizing angiotensin (e.g., DUP753) are known.

Summary of the Invention

Angiotensin IV (VAL-TYR-ILE-HIS-PRO-PHE),[1] a degradation product of angiotensin II previously thought to be inactive, interacts directly with endothelial cells to induce expression of PAI-1 and thereby to inhibit clot lysis by t-PA. Moreover, angiotensin IV apparently does not substantially interact with the known angiotensin II receptor, because it does not effect the various physiological changes (vasoconstriction, increased blood pressure, etc.) characteristic of angiotensin II. Further evidence of the direct involvement of angiotensin IV with PAI-1 is found in data showing that the PAI-1-enhancing effect of Angiotensin II is blocked when the conversion of angiotensin II to angiotensin IV is blocked. These findings have application in the following several aspects of the invention. Broadly, the invention can be divided into aspects featuring promoting fibrinolysis and aspects featuring inhibiting fibrinolysis.

Promoting Fibrinolysis

One aspect of the invention generally features methods of promoting fibrinolysis by administering to a patient an angiotensin IV antagonist, thereby reducing

[1] Angiotensin IV is sometimes referenced as A-IV.

expression of PAI-1. Angiotensin IV antagonists include substances that reduce the effect of (antagonize) angiotensin IV. For example, A-IV antagonists may compete with angiotensin IV for binding to the A-IV receptor, or they may directly bind angiotensin IV, rendering it inactive. One measure of such competition is the equilibrium dissociation constant (Kd) measure using the AT_4 receptor binding assay described below and in PCT WO 94/00492. For example, Kd may be $< 3 \times 10^{-6}$m, or, more preferably, $< 3 \times 10^{-8}$m, or, most preferably, $< 3 \times 10^{-9}$m. Specificity (e.g,. low ($> 10^{-6}$m) binding affinity for AT_1 and AT_2 receptors) is also preferred. Antagonism -- i.e., the substantial absence of PAI-1 induction -- can be determined by the substantial failure to induce PAI-1 mRNA in the PAI-1 induction assays described below, particularly in Examples 4 and 5.

Specific antagonists useful in this aspect of the invention include peptide analogs of angiotensin IV which: a) inhibit binding of angiotensin IV to a mixture containing an endothelial cell receptor specific for angiotensin IV, and b) do not themselves effect expression of PAI-1. One group of such compounds are analogs having properties a) and b) which have the

following general formula (or the acetate salts of those compounds):

(Formula I)

$$A - B - C - HIS - PRO - D$$

where A = SAR or VAL

B = TYR OR TRP

C = ILE or VAL and

D = ALA or PHE;

provided that if A is VAL and B is TYR and C is ILE, then D is ALA. VAL is preferred in position 1. As noted below, with compounds according to Formula I, the antagonism or inhibition may be direct or it may be as a pro-drug which itself lacks A-IV receptor binding but which is metabolized into an antagonist. Specific compounds within that general formula are given below.

Alternatively, the invention features antagonists which are polypeptide analogs of angiotensin IV which have properties a) and b) and have the following general formula:

(Formula II)

$$E - F - G - X_1 - X_2 - H$$

where:

E is a hydrophobic amino acid, preferably norleucine or benzylcysteine;

F is an aromatic amino acid (preferably tyrosine or iodotyrosine or an amino acid with a naphthalene side chain);

G is a hydrophobic non-aromatic amino acid, preferably norleucine, isoleucine, leucine or valine;

X_1 and X_2 are independent and can be any amino acid or non-amino acid or moiety containing a spacing function such as

$-(CH_2)_n-$, where n is 1-8;

H any hydrophobic amino acid (including D-PHE), except L-PHE.

Antagonists may also be compounds having properties a) and b) and having one of formulas III, IV, or V, below. Formula III is:

$$E - F - G - X_1 - X_2 - X_3$$

where

$G-X_1$ are joined by a non-peptide bond (preferably one with increased rotational freedom) as described below;

E, F, and G are as described for Formula II; and

X_1, X_2, and X_3 are any amino acid or spacing moiety as described above for X_1 and X_2 in formula II.

Formula IV is:

$$E - F - G - X_4$$

where E-F are bonded by a non-peptide linkage as described below (whether or not there is increased rotational freedom compared to a peptide bond);

E, F, and G, are as described for Formula II (preferably E is NorLeu or Val and F is TYR and G is ILE); and

and X_4 is optional, and can be -NH-Z where Z is -H; or $-(CH_2)n-NH_2$, where n=1-8; or another non-amino acid hydrophobic organic adduct (e.g., $X_1-X_2-X_3-$ where each of X_1, X_2, and X_3 is optional or is defined as above for Formula II).

Finally, the following formula can be used:

(Formula V)

$$J - K - L - X_5 - X_6 - X_7$$

where J = VAL or any hydrophobic amino acid;

K = TYR OR PHE;

L = any aliphatic amino acid; and

X_5, X_6, and X_7 = independently, any amino acid, preferably GLY. If J is VAL and K is TYR and L is ILE and X_5 is HIS and X_6 is PRO, then X_7 is not PHE.

It should be understood that the amino acids specified in the above formulas may be linked with peptide bonds or by non-peptide bonds. For example, residues (particularly $G-X_1$ in formula III) may be linked by bonds which provide more rotational freedom than a peptide bond, such as the called methylene bond isosteres in which the peptide bond [-(CO-NH)-] is replaced with a

-(CH_2-NH)- linkage. See generally, Sasaki and Coy, *Peptides*, 8:119-121 (1987). Other non-peptide bonds include amino-alcohol bonds [-CH=C(OH)-NH-] or other functions such as [-CH=C(OH)-N-] or reverse peptide bonds such as the one shown in the following example linking peptide residues X_2 and X_3: NH_2-X_1-CO-NH-X_2-NH-CO-X_3-COOH. Such non-peptide bonds can be used for one or more of the linkages in the antagonist. Specific compounds are discussed below.

Other antagonists include antibodies specific for angiotensin IV, particularly monoclonal antibodies or Fab fragments derived from such antibodies.

A second aspect of the invention generally features methods of promoting fibrinolysis by providing a therapeutically effective amount of a compound which inhibits production of angiotensin IV from angiotensin II. Preferred compounds are inhibitors of aminopeptidase A or aminopeptidase M, such as amastatin.

Medical indications for either of the first two aspects of the invention are those in which the patient has potentially injurious clot formation and fibrinolysis is therefore desired. Specific indications are listed below in this document.

A third aspect of the invention generally features compounds (particularly peptide analogs of angiotensin IV) that antagonize PAI-1 expression by antagonizing binding of angiotensin IV to its receptor. Such

compounds include those that fall within the general formula provided above that inhibit binding of angiotensin IV to endothelial cells, and that do not themselves effect expression of PAI-1. Such analogs include the compounds of Formula I, II, III, or IV, above.

The invention also features physiologically acceptable compositions comprising a therapeutic amount of an angiotensin IV antibody (preferably a monoclonal antibody) or an Fab fragment thereof.

The invention also features physiologically acceptable compositions comprising a therapeutic amount of an inhibitor of aminopeptidase A or aminopeptidase M, such as amastatin.

A fourth aspect of the invention features a method of screening candidate compounds for the ability to promote fibrinolysis by providing a mixture that includes angiotensin IV (or an angiotensin IV receptor-binding analog), an angiotensin IV-specific receptor, and the candidate compound. The screen is conducted by determining whether the candidate inhibits binding of angiotensin IV to the receptor. Candidate compounds include angiotensin IV muteins (e.g., conservative substitutions of angiotensin IV, mutations within the general formula given above, and others) and organic compounds designed therefrom by techniques that are generally known. In addition, such compounds include

antibodies (particularly monoclonal antibodies) specific for angiotensin IV, or Fab fragments derived from a such antibodies.

Inhibiting Fibrinolysis

A fifth aspect of the invention generally features methods of inhibiting fibrinolysis in a patient by providing a therapeutically effective amount of angiotensin IV or an agonist thereof. Angiotensin IV may be provided by administering angiotensin IV directly, by administering its immediate precursor, angiotensin III, or by administering or enhancing the activity of enzymes that convert precursors into angiotensin IV. Specific agonists that have application in this aspect of the invention are peptide analogs of angiotensin IV that operate on endothelial cells to bind and enhance PAI-1 expression by the assays described below. This aspect of the invention is specifically indicated for patients who have at least some ability to form clots, but who could benefit from additional clotting. Specific such conditions are listed in this document below.

The invention also features a physiologically acceptable composition comprising a therapeutically effective amount of angiotensin III or IV, or agonists thereof.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof and from the claims.

Brief Description of the Drawings

Fig. 1 is a diagram showing the degradation sequence of the peptides angiotensin I, II, III, and IV, as well as the peptides' sequences and inhibitors of the degratory enzymes.

Fig. 2 is a plot showing the binding of ^{125}I-Ang II to bovine aortic endothelial cells at room temperature, with total, non-specific and specific binding isotherms shown.

Fig. 3 is a Scatchard plot of specific binding data, yielding a D_d of approximately 2 nM.

Fig. 4 is a graph showing the effect of DTT on ^{125}I-Ang II (0.27 nM) to bovine aortic endothelial cells. Values represent the means $\pm$SD of one experiment performed in triplicate.

Fig. 5 is a plot showing the effect of saralasin (Sar^1,Val^5,Ala^8)-angiotensin on ^{125}I-AngII (0.5 nM) binding to bovine aortic endothelial cells. Values represent averages obtained from two experiments.

Fig. 6 is a dose response curve of PAI-1 secretion into the conditioned media of bovine aortic endothelial cells following exposure to Ang II.

Fig. 7 is a Northern blot showing the induction of PAI-1 RNA after treatment of bovine aortic endothelial cells with angiotensin II.

Fig. 8 is a graph showing the dose response of PAI-1 RNA induction by Ang II.

Fig. 9 is a Northern blot showing the time course of induction of PAI-1 RNA after treatment of bovine aortic endothelial cells with 20 nM angiotensin II.

Fig. 10 is a graph showing the PAI-1 mRNA levels as a function of time, in bovine aortic endothelial cells treated with 20 nM angiotensin II.

Fig. 11 is a graph of the mean BP at baseline and after increasing doses of Ang II alone, Ang IV alone, saline, and Ang II in rabbits pretreated with amastatin. Error bars represent SEM.

Fig. 12 is a graph of the mean plasma PAI-1 activity at baseline and after increasing doses of Ang II alone, Ang IV alone, saline, and Ang II in rabbits pretreated with amastatin. Error bars represent SEM.

Fig. 13 illustrates that although Ang II (10 nm) is capable of inducing PAI-1 expression in cultured endothelial cells, the effect of this peptide is not blocked by the presence of an AT_1 blocker (Dup 753) [1 μM] or by a T_2 receptor.

Fig. 14 shows the effect of Ang IV concentrations on PAI-1 expression by cultured endothelial cells. Confluent cultures of BAEC were washed, then incubated in serum-free DMEM in the presence of captopril (1 μM) overnight. The cells were then washed, and serum free media was replaced and incubated with vehicle or ANG IV for 8 hours at the indicated concentrations.

Figs. 15A and 15B show the duration and time course of ANG IV-induced PAI-1 expression by cultured endothelial cells. In Fig. 15A confluent cultures of BAEC were washed, then incubated in serum-free DMEM in the presence captopril (1 μM) overnight. The cells were then washed and incubated with vehicle or ANG IV for 1-6 hours. In Fig 15B, the time course of Ang IV induced increase of PAI-1 expression by BAEC was determined by densitometry and normalized using the 28S band.

Fig. 16 shows the effect of the aminopeptidase inhibitor Amastatin on Ang II-induced PAI-1 expression by BAEC. Confluent cultures of BAEC were washed, then incubated in serum-free DMEM in the presence of captopril (1 μM) overnight. The cells were then washed and incubated with vehicle or Ang II (10 nM) in the absence and presence of increasing concentrations of amastatin.

Fig. 17 shows the effect of the aminopeptidase inhibitor Amastatin on Ang IV-induced PAI-1 expression by BAEC. Confluent cultures of BAEC were washed, then incubated in serum-free DMEM in the presence of captopril (1 μM) overnight. The cells were then washed and incubated with vehicle or Ang IV (10 nM) in the absence and presence of increasing concentrations of amastatin.

Fig. 18 shows the effect of angiotensin receptor antagonists on Ang IV-induced PAI-1 expression by cultured endothelial cells. Confluent cultures of BAEC were washed, then incubated in serum-free DMEM in the

presence of captopril (1 μM) overnight. The cells were then washed and incubated with vehicle or Ang IV (10 nM) in the presence of the AT_1 receptor antagonist Dup 753 (1 μM), the AT_2 receptor antagonist PD123177 (1 μM), and the AT_4 receptor antagonist WSU 1291 (1 μM).

Fig. 19 shows the effect of the conversion of angiotensin on PAI-1 expression by cultured endothelial cells. Confluent cultures of BAEC were washed, then incubated in serum-free DMEM in the presence of captopril (1 μM) overnight. The cells were then washed and incubated with vehicle or Ang I (10 nM) in the absence or presence of captopril (1 μM), Ang II (10 nM) in the absence or presence of amastatin (1 μM), Ang III (10 nM) in the absence or presence of amastatin (1 μM), or Ang IV (10 nM).

Description of Preferred Embodiments

Embodiments of the aspects of the invention related to enhancing PAI-1 levels and thereby decreasing fibrinolysis will be described separately from embodiments related to reducing PAI-1 levels and thereby increasing fibrinolysis.

I. Promoting Fibrinolysis

The aspect of the invention related to promoting fibrinolysis features decreasing available angiotensin IV or antagonizing the activity of angiotensin IV. For example, one may competitively antagonize A-IV interaction with its specific endothelial cell receptor.

Compounds useful for antagonizing angiotensin IV include those which competitively bind to the angiotensin IV receptor by the assay described below, but do not induce PAI-1 expression. Candidate peptide antagonists are discussed above with regard to Formula I. Specific analogs include the following compounds or the acetate salts of the following:

VAL-TYR-ILE-HIS-PRO-ALA;

SAR-TYR-ILE-HIS-PRO-ALA;

SAR-TYR-VAL-HIS-PRO-ALA;

VAL-TRP-ILE-HIS-PRO-ALA;

VAL-TRP-VAL-HIS-PRO-ALA;

SAR-TRP-VAL-HIS-PRO-ALA;

SAR-TYR-VAL-HIS-PRO-PHE;

SAR-TYR-ILE-HIS-PRO-PHE;

SAR-TRP-VAL-HIS-PRO-PHE; or

SAR-TRP-ILE-HIS-PRO-PHE.

Compounds of Formula 1 (particularly those with SAR in position 1) may not be antagonists in their own right, as determined by *in vitro* screening for AIV receptor binding. They nevertheless can be screened in *in vivo* models as pro-drugs which are metabolized to yield antagonists. VAL-TYR-ILE-HIS-PRO-ALA is an antagonist in its own right, and other compounds with VAL in position 1 may also be antagonists in their own right.

As noted above, compounds of general formulas II, III, and IV, above, which are A-IV antagonists can be

used. Some structure function guidelines are provided in PCT/US93/06038 (WO 94/00492), cited above. In each of Formula II-IV, it is preferred that E be norleucine and F be TYR. Accordingly, preferred compounds are NOR-TYR-G-X_4 as defined for Formula IV. Specific compounds antagonists include:

1. Divalinal angiotensin IV [$^{+}H_3N$-Val(CH_2NH)Tyr-Val(CH_2NH)-His-Pro-Phe-COO^{-}];

2. D-Val_1-angiotensin IV (i.e., angiotensin IV with a D-valine residue in the 1 position).

3. Norleucine-TYR-ILE-GLY-GLY-DPHE.

4. Norleucine-TYR-ILE-HN-$(CH_2)_6$-NH_2.

Candidate antagonists can be screened by the general methods described herein. Specific, non-limiting examples of such screens are given below in the Examples.

Clinical indications for treatment with the compounds of the invention include any medical conditions whose treatment would be benefitted by promoting fibrinolysis. These conditions include: a) thromboembolic disorders, b) prophylaxis of undesired clotting as a result of surgery, c) post-surgical maintenance of grafts or prostheses, d) congestive heart failure, e) cardiomyopathy, f) myocardial infarction, and g) cerebrovascular disease. Specific indications include: acute venous thrombosis, pulmonary embolism, atherosclerosis, ventricular or atrial thrombi, peripheral or mesenteric arterial thrombosis, acute

coronary infarction or occlusion, and acute peripheral artery occlusion. The compounds may also be administered as prophylaxis against thromboemboli associated with major surgery, congestive heart failure, cardiomyopathy, myocardial infarction, pregnancy, or disseminated intravascular coagulation.

II. Inhibiting Fibrinolysis

The aspects of the invention relating to decreasing fibrinolysis involve enhancing levels of angiotensin IV or agonists thereof. Angiotensin IV may be provided by administering angiotensin IV directly, by administering its immediate precursor, angiotensin III, or by administering or enhancing the activity of enzymes that convert precursors into angiotensin IV.

Specific agonists that have application in this aspect of the invention are peptide analogs of angiotensin IV that operate on endothelial cells to bind and enhance PAI-1 expression. Again, structure function relationships are provided in WO94/00492, cited above. A-IV agonists include: Lys_1-angiotensin IV; and $NorLeu_1$angiotensin IV (NorLeuYIHPF). Particularly preferred are the above agonists in which NorLeu is joined to the second residue by a non-peptide bond such as one of the above-described linkages: $-(CH_2)-NH-$. Candidate agonists may be screened for the ability to induce PAI-1 expression by the screens described below.

This aspect of the invention also features administering one or more enzymes that enhance the formation of angiotensin IV by natural pathways. Two such enzymes are aminopeptidase A and aminopeptidase M.

This aspect of the invention is specifically indicated for patients who have at least some ability to form clots, but who could benefit from additional clotting. Specific such conditions include the following: afibrinogenemia, dysfibrinogenemia, hypoprothrombinemia, parahemophilia, hypoconvertinemia, hemophilia A, hemophilia B, Stuart-Prower factor deficiency, plasma thromboplastin antecedent deficiency, Hageman trait, thrombocytopenia, disorders of platelet function, Von Willebrand's disease, hepatic dysfunction, circulating anticoagulants, inherited defects in natural coagulation inhibitors (such as antithrombin, protein C, or protein S), dysplasminogenemia, defective release or diminished venous content of plasminogen activator, excessive release of PAI, heparin cofactor II deficiency, homocystinuria, chronic congestive heart failure, metastatic tumor or malignancy, extensive trauma or major surgery, myeloproliferative disorders, or treatment with oral contraceptives or L-asparaginase.

III. General Aspects of the Invention

The various peptides described herein may be synthesized by various well known techniques including solid phase synthesis and synthesis by cells engineered to contain recombinant nucleic acid expressing the desired peptide. Angiotensin III or IV and some other peptides also may be purchased from vendors such as Bachem, Torrence, CA; Clonetech, Palo Alto, CA; Sigma, St. Louis, MO. Illustrative synthetic techniques are provided below, and those skilled in the art will understand that the same general techniques may be used to synthesize other compounds according to the invention.

Aminopeptidase A and aminopeptidase M may be obtained by the general methods of Kugler, *Histochemistry* (1982) 74:247-261; Hui, *J. Biol. Chem.* 267:6613-6618; , or from vendors such as Cal Biochem, San Diego, CA; or Boheringer Mannheim, Indianapolis, IN.

Antibodies to angiotensin IV may be obtained by standard techniques involving challenging a mammal (e.g. a mouse, rat, rabbit) with angiotensin IV and recovering polyclonal antibodies from serum or recovering antibody producing cells, immortalizing them and screening for clones producing the desired antibody.

The term "patient" means any mammalian patient to which inhibitors or promoters of fibrinolysis may be administered. Patients specifically intended for treatment with the method of the invention include

humans, as well as nonhuman primates, sheep, horses, cattle, goats, pigs, dogs, cats, rabbits, guinea pigs, hamsters, gerbils, rats and mice, as well as the organs, tumors, and cells derived or originating from these hosts.

A therapeutically-effective amount of compound is that amount which produces a result or exerts an influence on the particular condition being treated and to be safe and effective in treating the condition of either excess or deficient fibrinolysis for which the compound is administered. Those skilled in the art will understand that dosages can be optimized for a given medical indication and a given therapeutic by standard techniques such as establishing a dosage in an animal model, predicting a subtherapeutic dose for humans, testing safety in humans by increasing that subtherapeutic dose, and then optimizing the therapeutic dose.

The compounds of the invention may be administered in any manner which is medically acceptable. This may include injections, by parenteral routes such as intravascular, intravenous, intra-arterial, subcutaneous, intramuscular, intratumor, intraperitoneal, intraventricular, intraepidural, or others, as well as oral, nasal, ophthalmic, rectal, topical, or as an inhalant preparation. Sustained release administration is also specifically included in the invention, by such

means as depot injections or erodible implants. The compounds may also be directly applied during surgery.

It should be understood, however, that the foregoing description of the invention is intended merely to be illustrative by way of example only and that other modifications, embodiments, and equivalents may be apparent to those skilled in the art without departing from its spirit. The following experiments are recited to illustrate the invention, not to limit it. Examples 1 and 2 relate to studies of the binding of angiotensin II (AngII) and of the effect of such binding on endothelial cells. Examples 3 and 4 relate to identification of the moiety responsible for inducing expression of PAI-1.

EXPERIMENTS

Example 1 - Characterization of the binding of angiotensin II to cultured endothelial cells.

We first studied the role of angiotensin (Ang) II in the regulation of endothelial fibrinolysis, by determining if Ang II binds to the target cell, which in this case was bovine aortic endothelial cells. In these experiments, we used early passage bovine aortic endothelial cells (P2-4) grown to confluence in DMEM supplemented with penicillin, streptomycin, and 10% newborn calf serum. When these experiments are performed at room temperature, we have found that binding equilibrium occurs by 45 min. Further, the binding characteristics of cells > passage 5 is highly variable.

As shown in Fig. 2, ^{125}I-Ang II appears to bind to bovine aortic endothelial cells in a saturable and specific manner. A Scatchard transformation of the specific binding data (Fig. 3) yields an apparent K_d of ~2 nM, with B_{max} = 105 fmol/10^6 cells.

We further showed that the endothelial angiotensin receptor differs from the classic AT_1 receptor in several important ways. After exposing bovine aortic endothelial cells to 5 mM DTT, we observed a 50% reduction in total binding (Fig. 4). This suggests that the bovine aortic endothelial cells receptor for Ang II is somewhat less sensitive to the effects of reducing agents that the classic AT_1 receptor. To test whether the Ang II receptor on the bovine aortic endothelial cells is similar or identical to the classic AT_1 receptor, we measured the potency of the antagonist saralasin for binding. It is a well established property of the AT_1 receptor that the antagonist saralasin binds with a K_i=0.4 nM. We performed an experiment in which unlabeled saralasin was used to displace ^{125}I-AngII from bovine aortic endothelial cells and found an IC_{50} @10 μM (Fig. 5). The failure of saralasin to compete effectively for binding to bovine aortic endothelial cells provides additional evidence that the receptor we are dealing with is not a classic AT_1, and in this aspect it appears quite similar to the binding site for Ang IV.

Example 2 - Ang II induces the production and secretion of PAI-1 by bovine aortic endothelial cells.

In these experiments, we investigated the effects of Ang II on the secretion of PAI-1 into the media of cultured endothelial cells. Exposure of bovine aortic endothelial cells to Ang II appears to induce the secretion of PAI-1 in a dose-dependent manner, as shown in Fig. 6. Ang II (in the concentrations indicated) was added to washed, confluent cultures of bovine aortic endothelial cells, and the conditioned media was removed for assay after 18 hours. Levels of PAI-1 antigen in the media were determined using a specific ELISA for PAI-1, such as the assay described in DeClerk et al., *Blood* (1988) 71:220-225, or the assay sold by Biopool, AB, Umea, Sweden.

The EC_{50} for this response is ~20 nM, and corresponding changes in PAI-1 activity (after reactivation of the latent protein with guanidine hydrochloride) have also been observed. This effect of Ang II on PAI-1 appears to be specific, as we have not observed a similar increase in t-PA antigen or activity in the conditioned media. The increases in PAI-1 activity and antigen observed thus far have been associated with an approximate 4-fold increase in PAI-1 message (Figs. 7 and 8). The cells for these experiments were incubated for 8 hours in fresh serum-free media containing the indicated concentrations of Ang II. Total

RNA was extracted and analyzed by Northern blotting. Fig. 8 shows levels of PAI-1 mRNA as quantitated by slot/blot analysis in triplicate and normalized for signal intensity of control probe for β-actin. This stimulatory effect of Ang II on PAI-1 mRNA levels does not appear to be prevented by Dup 753 (1 μM) or by saralasin (1 μM).

Ang II is also associated with a time-dependent increase in PAI-1 mRNA that peaks 6-8 hrs after exposure to Ang II, as assessed by Northern blot analysis (Fig. 9).

Confluent cultures of bovine aortic endothelial cells were incubated with 20 nM Ang II in serum-free DMEM. Total cytoplasmic RNA was extracted at the indicated times and analyzed by Northern blotting (Fig. 9). The results corresponding to the 3.3-kb PAI-1 mRNA species were quantified by transmission densitometry. To control for variability in gel loading, the blots were stripped and rehybridized with a cDNA probe for β-actin. Normalized data are plotted in the Fig. 10.

In the experiments described above we have first demonstrated that Ang II binds to bovine aortic endothelial cells in a specific and saturable manner. Second, this binding appears to have a functional effect on bovine aortic endothelial cells in inducing the synthesis of PAI-1 message and the secretion of PAI-1 into the conditioned media. Importantly, we have

observed increased levels of PAI-1 mRNA induced by physiologically relevant doses of Ang II (i.e. 0.1 nM). The potential significance of these in vitro findings are supported by recent data obtained during the infusion of Ang II into healthy human volunteers. In these studies, normotensive volunteers (N=4) received an infusion of Ang II in graded doses of 1.0, 3.0. and 10.0 ng/kg/min., while 4 additional normotensive volunteers received an infusion of D5W. Plasma levels of t-PA and PAI-1 were measured prior to and at the conclusion of each dose. Plasma PAI-1 levels increased in the subjects that received the Ang II infusions in a dose-dependent manner, from 14.7 $\pm$ 5.3 to 33.5 $\pm$ 10.6, (mean values $\pm$ SEM, $p<0.001$ by ANOVA). Similar findings have been obtained in an additional group of hypertensive volunteers (N=6) that received a constant infusion of Ang II (3 ng/kg/min) over a 45 min period. In these individuals, we observed a 60% increase in plasma PAI-1 compared with pretreatment levels ($p<0.04$).

These data indicate that Ang II selectively increases levels of PAI-1 in plasma, while plasma t-PA levels do not increase.

Example 3 - Identification of agent responsible for enhancing PAI-1

We have also undertaken a series of experiments designed to identify the specific form of angiotensin that is responsible for the increased plasma PAI-1 levels

seen in our human studies. We examined the effects of graded infusions of Ang II and Ang IV on blood pressure and plasma PAI activity in healthy New Zealand White rabbits. As shown in Fig. 11, the infusion of Ang II was associated with a dose-dependent increase in mean blood pressure, while the animals that received Ang IV exhibited a stable mean BP throughout the course of the experiment. In contrast with these divergent effects on blood pressure, both agents induced a dose-dependent increase in plasma PAI-1 levels (Fig. 12). Control animals that received an infusion of normal saline showed no changes in BP or plasma PAI-1, while animals that received Ang II after pretreatment with amastatin (a synthetic inhibitor of the aminopeptidase that is responsible for the conversion of Ang III to Ang IV) exhibited the expected increases in blood pressure while the PAI-1 response was reduced considerably. These data add further evidence that Ang IV is responsible for the induction of endothelial PAI-1 production and secretion.

Example 4 - Method of In Vitro Testing of Angiotensin IV Antagonists

A variety of compounds are suitable for administration in the methods of the invention. These compounds will each have angiotensin IV agonistic or antagonistic activity. Methods of screening these compounds are presented below.

Northern blotting studies

Cultured endothelial cells are exposed to Ang IV in the presence and absence of antagonists. After a several hour incubation period, cells are lysed and total RNA is isolated, gel electrophoresed, and transferred to a nylon membrane. The relative expression of PAI-1 mRNA is determined by hybridizing the membranes with ^{32}P-labeled cDNA probes specific for PAI-1. Results are quantified by autoradiography. Potency of Ang IV antagonism is inversely proportional to induction of PAI-1 message.

Protein secretion

Cultured endothelial cells are exposed to Ang IV in the presence or absence of antagonists. After a 24 hour incubation period, the conditioned media is removed, centrifuged, and aliquots of the supernatant are assayed for the presence of PAI-1 antigen using a specific enzyme-linked immunosorbent assay (ELISA) for PAI-1.

Example 5 - Method of In Vivo Testing of Angiotensin IV Antagonists

New Zealand White rabbits are injected with graded doses of Ang IV. Simultaneously, they receive intravenous infusion of an ANG IV antagonist. Venous blood samples are collected during the infusions and assayed for PAI-1 antigen using a specific ELISA. Effective antagonists will blunt the secretion of PAI-1 into plasma.

Example 6 - Further Characterization of Endothelial Cell Response

The following experiments provide additional characterization of the physiological response to A-IV receptor binding. We first describe the materials and methods used to further characterize the endothelial cell response to A-IV antagonists or agonists.

Fetal bovine serum (FBS) and bovine calf serum (BCS) were obtained from Hyclone laboratories, Logan, UT. Tissue culture media was from Gibco BRL, Gaithersburg, MD. Endothelial mitogen and Dil-acetylated LDL (Dil-Ac-LDL) were obtained from Integrated Biotechnology, Stoughton, MA. Amastatin, bestatin, gelatin, penicillin, streptomycin, antibody against von Willebrand factor and trypsin/EDTA were from Sigma Chemical Co., St. Louis, MO. Ang I, Ang II, Ang III and Ang IV, were from Bachem, Torrance CA. Dup 753 was kindly provided by Ronald D. Smith, Ph.D., DuPont Pharmaceutical Co., Wilmington, DE, and PD123177 (available from Parke Davis Pharm.). Divalinal angiotensin (WSU1291) was synthesized as described below. [^{32}P]dUTP was from New England Nuclear, Boston, MA.

The following cell culture techniques were used. Bovine aortic endothelial cells (BAEC) were obtained from fresh bovine aortas[2] and harvested using 0.1%

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[2] See generally Vaughan et al., *J. Clin. Invest.* 95:995-1001 (1995)

collagenase. After they reached confluence, cells were detached using trypsin/EDTA, pooled, and serially propagated in Dulbecco's Modified Eagles Media (DMEM) supplemented with penicillin (50 units/ml), streptomycin (50 μg/ml) and 20% BCS. The cells were incubated in humidified 95% air/5% CO_2 at 37°C. The cells exhibited typical morphological features of endothelial cells and immunofluorescence staining against antibodies to von Willebrand factor and by the uptake of DiI-Ac-LDL. Cells of passage number 1 were exclusively utilized in these experiments. BAEC were grown to confluence in 100-mm tissue culture dishes, washed twice with sterile PBS and then incubated overnight in serum-free DMEM containing 1.0 μM captopril to minimize autocrine angiotensin effects. The cells were washed with fresh serum-free DMEM (in the absence of captopril) and exposed to Ang I, Ang II, or Ang IV in the presence or absence of the AT_1 receptor antagonist Dup 753 (1.0 μM), a highly specific antagonist of the AT_2 receptor PD123177 (1.0 μM), or the AT_4 receptor antagonist, WSU1291 (1.0 μM) for 6 hours.

RNA from cells was isolated and measured as follows. Total cellular RNA was prepared from confluent cultures of BAEC by the acid guanidium thiocyanate-method, disclosed in Chanczynski et al., *Anal. Biochem.* 162:156-159, followed by isopropanol precipitation (RNAzol, Cimna Biotecx, Houston, TX). RNA pellets were resuspended in DEPC-treated H_2O and their

concentrations determined by absorbance at 260 nm. The relative amounts of specific mRNA present was quantified by Northern hybridization using specific riboprobes. RNA (18 μg) was size fractionated on 1.2% formamide agarose cells and transferred to nylon membranes (Zeta probe®: Bio- Rad Laboratories, Richmond, CA) and crosslinking was performed under ultraviolet light, with exposure to 254 nm for 30 seconds at 1.5 J/cm^3 (Bio-Rad Laboratories). The membranes were prehybridized overnight at 60°C in a mixture of 50% formamide/5 X SSC/5 X Denhardt's solution 1% SDS containing sonicated, heat-denatured, salmon sperm DNA (200 μg/ml). Membranes were hybridized overnight with PAI-1 riboprobes labelled with [^{32}P]dUTP at 60°C in a shaking water bath, washed using 0.2 X SSC and 0.1% SDS, initially at room temperature for 30 minutes X2, then at 68°C in 0.1x SSC, 0.1% SDS for 30 minutes, air dried and exposed to Kodak XAR film with intensifying screen at -70°C. Relative RNA loading was determined by examination of ethidium-stained gels. Reflectance densitometry of the ethidium-stained 28S bands was used for normalizing autoradiographic data.

The cDNA Riboprobes used are as follows. The cDNA template for this probe consisted of an 600 bp fragment containing nucleotides 389 to 994 of human PAI-1 (GENEBANK). Complementary 0.6 kb mRNA transcripts to PAI-1 were generated using a commercially available kit (maxiscript™, Ambion, Inc., Austin, TX). The T7 phage

RNA polymerase was utilized for the *in vitro* synthesis of RNA transcripts from the DNA template.

The effect of Ang II on PAI-1 mRNA Expression was determined as follows. After pretreatment with captopril (10 μM), BAEC were exposed to Ang II (10 mM) for 6 hours in the absence and presence of specific antagonists of the AT_1 and AT_2 receptor subtypes (Dup 753, 1 μM and PD123177, 1 μM, respectively. At the end of the exposure period the cells were washed, solubilized and total RNA was extracted as described. Fig. 13 demonstrates the results of Norther blot analysis using a riboprobe to PAI-1. In control cells, PAI-1 mRNA was expressed at low, but detectable, levels. Exposure of the cells to Ang II resulted in the increased expression of PAI-1 message. The degree of expression of PAI-1 was unaltered by a 100-fold excess of Dup 753 or PD123177.

The effect of Ang IV on PAI-1 mRNA expression was determined as follows. In these experiments confluent cultures of BAEC were exposed to Ang IV over the concentration range 0 to 10 nM (Fig. 14). Compared with vehicle treated controls, AIV (10 nm) induced Ang IV resulted in a dose dependent increase in the expression of PAI-1. On average, a 5.3 ± 2.6-fold (mean standard error, SEM) increase in PAI-1 mRNA levels. A time dependent effect on Ang IV on PAI-1 expression was also demonstrated (Figs. 15A and 15B). The induction of PAI-1

mRNA expression produced by Ang II is evident within two hours and is maximal at 4 hours.

The effect of Amastatin on the response to Ang II and Ang IV was determined as follows. Amastatin is a potent inhibitor of endopeptidases[3] and prevents the conversion of Ang II to smaller fragments including Ang IV. To investigate the role these aminopeptidases play in regulating the induction of PAI-1 mRNA levels by Ang II, cells were exposed to Ang II (10 nM) in the presence of increasing concentrations of amastatin (0-1,000 nM). As demonstrated in Fig. 16, amastatin resulted in dose-dependent reduction in the expression of PAI-1 mRNA. In contrast, amastatin did not alter the expression of PAI-1 following exposure of the cells to Ang IV (10 nM, Fig. 17). In the presence of amastatin (1 μM), the PAI-1 mRNA signal was 80% of that seen in untreated control cells. In contrast, the PAI-1 mRNA expression in cells treated with Ang IV and amastatin (1 μM) was 3.6-fold greater than controls.

Competitive Inhibition of Ang II Induction of PAI-1 Expression in shown in Fig. 18. The compound WSU1291 is a potent partial non-peptide inhibitor of the AT_4. The relative potencies of specific antagonists of the AT_1, AT_2, and AT_4 receptor subtypes (Dup753, PD123177 and WSU1291, respectively) on the endothelial cell response

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[3] Rich et al., *J. Med. Chem.* 27:418-422 (1984)

to Ang IV is illustrated in Fig. 18. The AT_1 and AT_2 receptor antagonists were generally less effective than WSU1291 (1 μM) in preventing the Ang IV (10 nM) stimulated increase in PAI-1 mRNA levels. In contrast, cells treated with the combination of WSU1291 (1 μM) and Ang IV (10 nM) exhibited PAI-1 mRNA levels that are effectively comparable to the untreated control. PD123177 (1 μM) resulted in a 6±5% (n=3) reduction in the response to Ang IV. Dup 753 (1 μM) was associated with a 16±8% (n=4) increase in the expression of PAI-1 mRNA.

Identification of Ang IV as the requisite peptide for PAI-1 expression is shown in Fig. 19. The renin angiotensin system is comprised of a sequence of reactions that results in the formation of peptide fragments of angiotensin which differ in their composition of functional precursors. In this experiment, a series of angiotensin peptides was examined for its ability to induce PAI-1 expression in the presence or absence of selective peptidase inhibitors, as demonstrated in Fig. 19. Captopril (10 μM), which prevents the conversion of Ang I to Ang II, also blocked the induction of PAI-1 expression. Administration of Ang II (10 nM) resulted in expression of PAI-1, but this effect was blunted by amastatin (1 μM). A similar response to Ang III (10 nM) in the absence and presence of amastatin (1 μM) were obtained. Finally, it is again demonstrated that the hexapeptide Ang IV, is sufficient

to stimulate the expression of PAI-1. As demonstrated in Fig 17, amastatin had no effect on the response of endothelial cells to Ang IV.

Thus, AIV induces an increase in the expression of PAI-1 in cultured BAEC. This response exhibits both a time and a dose dependence and it appears that angiotensin is not capable of inducing endothelial PAI-1 mRNA expression until it is converted to the hexapeptide Ang IV.

AIV induced PAI-1 mRNA expression can be blocked by a specific AT_4 receptor antagonist (WSU1291). Importantly, this effect of the AT_4 receptor antagonist appears to be specific and not due to undefined general effects on cellular function, since endothelial cells retain their morphologic characteristics and proliferative capacity when cultured in the presence of WSU1291 (data not shown).

Example 7 - Synthesis of Divalinal Angiotensin IV

Non-peptide angiotensin IV analogs having methylene bond isosteres ($-CH_2-NH-$) were synthesized using the racemate free amino aldehyde synthesis, Schiff's base formation, and reduction with sodium cyanoborohydride. For example, synthesis of $^{+}H_3N$-Val(CH_2NH)Tyr-Val(CH_2NH)-His-Pro-Phe-COO^- (designated divalinal AIV) was accomplished by utilizing standard solid phase protocols with t-BOC protected amino acids and amino aldehydes. The same general protocol is used

to produce other AIV ligands with methylene bonds between desired amino acid residues using the appropriate amino acid aldehyde as a reagent. R-group protection was: Tosyl for His and 2,6-dichlorobenzyl for Tyr. Synthesis occurred on a t-Boc-Phe substituted resin (0.76mmol/gram of 1% cross-linked divinyl benzene resin from Peninsula).

For amino acid coupling the following protocol was used: methylene chloride wash: 1X1 min; 45% w/v trifluoroacetic acid and 0.08% indole in methylene chloride deprotection: 1X3 min and 1X30 min; methylene chloride wash: 5X1 min; isopropanol wash: 3X1 min; methylene chloride wash: 10% v/v triethylamine in methylene chloride neutralization: 1X1 min and 1X5 min; methylene chloride wash: 2X1 min; isopropanol wash: 2X1 min; methylene chloride wash: 2X1 min: isopropanol wash: 2X1 min; methylene chloride wash: 3X1 min; amino acid coupling with a 2.5 or 5-fold excess of amino acid and EDC in methylene chloride: reaction times of 1.5 to 3.5 hours; methylene chloride wash: 3X1 min; isopropanol: 3X1 min; methylene chloride wash: 3X1 min. The above protocol was repeated for each cycle. Re-links of animo acids repeated all steps beginning with the neutralization. All linkages and deprotections were monitored with Kasier ninhydrin test. Acylations less than 94% were repeated.

Valinal (N-t-BOC-L valine aldehyde from Peninsula) was linked to the free amino-terminal of the growing

peptide by formation of a Schiff's base intermediate with subsequent bond reduction. For this reaction the above protocol was utilized with the following alterations; prior to coupling, the resin was washed with dimethyl formamide 3X1 min; a 5-fold excess of valinal was added in 1% acetic acid/dimethyl formamide; a 10-fold mole ratio excess of sodium cynoborohydride (Sigma) was dissolved in 3ml 1% acetic acid/dimethyl formamide and added in equal aliquots at 0, 3, 5, 10, 15, 20, 25, 30, 40 and 50 min with concurrent nitrogen purge; the coupling was allowed to continue for 70 additional min; the resin was washed with dimethyl formamide 3X1 min. Linkage was assessed with the Kaiser test and revealed a slightly reddish color of the beads when greater than 94%.

The finished N-terminal deprotected resin-linked peptide was cleaved from the resin and side chain deprotected with anhydrous HF containing 10% anisole a 0°C for 40 min. The HF and anisole were removed under vacuum and the peptide washed in anhydrous ether. The peptide was extracted with 20% glacial acetic acid and lyophilized. The crude peptide was then purified by preparative reversed phase HPLC in two steps, the first an isocratic method using acetonitrile:triethylamine-phosphate, pH3 followed by a second gradient method using acetonitrile:water (0.1% TFA). The purified product was analyzed by analytical reversed phase HPLC

(acetonitrile:triethylamine-phosphate, pH3) gradient method (12-18% over 60 min at 2ml/min).

Replacement of the R_1-R_2 peptide bond with the methylene bond reduced affinity of binding to the AT4 receptor by 5-fold. Double replacement of both the R_1-R_2 and the R_3-R_4 peptide bonds and substitution of the R_3 Val with Ile produced the peptide: N-V_1-CH_2-NH-Y_2V_3-CH_2-NH-$H_4P_5F_6$-C (Divalinal AIV) that had equal or better affinity than AIV for the AT4 receptor. In addition, divalinal AIV has been shown to exhibit enhanced metabolic stability and to be a potent antagonist of AT4 receptor activity. Fig. 11 illustrates the comparative stability of ^{125}I-AIV and ^{125}I-Divinal AIV following exposure to a membrane fraction prepared from rat kidney. Kidney was chosen as the tissue of study because of its well-known degradative capacity. The metabolism of ^{125}I-Dival AIV by rat kidney membranes was determined as follows: Rat membranes (25 μg protein) were incubated with .6nM ^{125}I-peptide at room temperature in a buffer containing Tris, 50mM, pH7.4; NaCl, 150mM; BSA, 0.1%; EDTA, 5mM; bestatin, 20 μM; and Plummer's inhibitor, 50 μM. Metabolism was stopped by the addition of acetonitrile (final concentration 50%), and the samples were analyzed by reverse phase (C^{18}) HPLC. Fig. 11 of the above-referenced WO94/00492 shows that AIV is rapidly degraded while Dival AIV generally remains intact after 4 hours of incubation.

Other embodiments are within the following claims. For example, the claimed agonists and antagonists may be used for other medical indications such as those disclosed in PCT/US93/06038 (WO94/00492).

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WASHINGTON STATE UNIVERSITY
RESEARCH FOUNDATION

(ii) TITLE OF INVENTION: ANGIOTENSIN IV AND ANALOGS AS REGULATORS OF FIBRINOLYSIS

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(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 6

(B) TYPE: amino acid

(C) STRANDEDNESS: N/A

(D) TOPOLOGY: linear

(ix) FEATURE:

(D) OTHER INFORMATION: Xaa in position 1 could be Ser or Val; Xaa in position 2 could be Tyr or Trp; Xaa in position 3 could be Ile or Val; Xaa in position 6 could be Ala or Phe.

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

Xaa Xaa Xaa His Pro Xaa
1 5

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 6

(B) TYPE: amino acid

(C) STRANDEDNESS: N/A

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Val Tyr Ile His Pro Ala
1 5

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 6

(B) TYPE: amino acid

(C) STRANDEDNESS: N/A

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

Ser Tyr Ile His Pro Ala
1 5

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 6

(B) TYPE: amino acid

(C) STRANDEDNESS: N/A

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

Ser Tyr Val His Pro Ala
1 5

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 6
(B) TYPE: amino acid
(C) STRANDEDNESS: N/A
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

Val Trp Ile His Pro Ala
1 5

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 6
(B) TYPE: amino acid
(C) STRANDEDNESS: N/A
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

Val Trp Val His Pro Ala
1 5

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 6
(B) TYPE: amino acid
(C) STRANDEDNESS: N/A
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

Ser Trp Val His Pro Ala
1 5

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 8:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 6
(B) TYPE: amino acid
(C) STRANDEDNESS: N/A
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

Ser Tyr Val His Pro Phe
1 5

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 9:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 6
(B) TYPE: amino acid
(C) STRANDEDNESS: N/A
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

Ser Tyr Ile His Pro Phe
1 5

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 10:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 6
(B) TYPE: amino acid
(C) STRANDEDNESS: N/A
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

Ser Trp Val His Pro Phe
1 5

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 11:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 6
(B) TYPE: amino acid
(C) STRANDEDNESS: N/A
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

Ser Trp Ile His Pro Phe
1 5

What is claimed is:

1. A method of promoting fibrinolysis in a patient comprising administering to the patient a therapeutically effective amount of an antagonist of angiotensin IV.

2. A method of promoting fibrinolysis in a patient comprising administering to the patient a therapeutically effective amount of a compound which inhibits the conversion of angiotensin II to angiotensin IV.

3. The method of claim 1 or claim 2, wherein the patient is characterized by a medical indication selected from the group consisting of: a) thromboembolic disorders, b) prophylaxis of undesired clotting as a result of surgery, c) post-surgical maintenance of grafts or prostheses, d) congestive heart failure, e) cardiomyopathy, f) myocardial infarction, and g) cerebrovascular disease.

4. The method of claim 1 or claim 2 wherein the patient is characterized by myocardial infarction.

5. The method of claim 1 or claim 2 wherein the patient is characterized by cerebrovascular disease.

6. The method of claim 1 or claim 2 wherein the patient is characterized by a venous thromboembolic disorder.

7. The method of claim 1, wherein the antagonist of angiotensin IV is a compound having the following general formula, or an acetate salt thereof:

A - B - C - HIS - PRO - D

where A = SAR or VAL
B = TYR OR TRP
C = ILE or VAL and
D = ALA or PHE;

provided that if A is VAL and B is TYR and C is ILE, then D is ALA.

8. The method of claim 7 in which the antagonist of angiotensin IV is selected from the group consisting of the following compounds and acetate salts thereof:

VAL-TYR-ILE-HIS-PRO-ALA;
VAL-TRP-ILE-HIS-PRO-ALA; and
VAL-TRP-VAL-HIS-PRO-ALA.

9. The method of claim 1, wherein the antagonist of angiotensin IV comprises an antibody specific for angiotensin IV or an Fab fragment derived from such an antibody.

10. The method of claim 1 wherein the antagonist of angiotensin IV is a polypeptide analog of angiotensin IV that inhibits binding of angiotensin IV to its receptor.

11. The method of claim 10 wherein the antagonist of angiotensin IV is a compound having one of the following general formulas A-D:

(Formula A)

$$E - F - G - X_1 - X_2 - H$$

where:
E is a hydrophobic amino acid, preferably norleucine or benzylcysteine;
F is an aromatic amino acid (preferably tyrosine, iodotyrosine) or naphthalene;

G is a hydrophobic non-aromatic amino acid, preferably norleucine, isoleucine, leucine or valine;
X_1 and X_2 are independent and can be any amino acid or non-amino acid or moiety containing a spacing function such as $-(CH_2)_n-$ where n is 1-8;
H any hydrophobic amino acid (including D-PHE), except L-PHE;

(Formula B)

$$E - F - G - X_1 - X_2 - X_3$$

where
$G-X_1$ are joined by a non-peptide bond (preferably one with increased rotational freedom) as described below;
E, F, and G are as described for Formula A; and
X_1, X_2, and X_3 are any amino acid or spacing moiety as described above;

(Formula C)

$$E - F - G - X_4$$

where E-F are bonded by a non-peptide linkage as described below;
E, F, and G, are as described for Formula A; and
and X_4 is optional, and can be -NH-Z where Z is -H, $-(CH_2)n-NH_2$, where n=1-8, or other non-amino acid hydrophobic organic adduct, including $X_1-X_2-X_3-$ where each of X_1, X_2, and X_3 is optional or as defined for Formula A;

(Formula D)

$$J - K - L - X_5 - X_6 - X_7$$

where
J = VAL or any hydrophobic amino acid;
K = TYR OR PHE;
L = any aliphatic amino acid; and

X_5, X_6, and X_7 = any amino acid, preferably GLY; and, provided that, if J is VAL and K is TYR and L is ILE and X_5 is HIS and X_6 is PRO, then X_7 is not PHE.

12. The method of claim 11 in which E or J is norleucine and F or K is Trp.

13. The method of claim 11 in which G or L is Ile.

14. The method of any one of claims 11-13 in which the antagonist includes at least one non-peptide bond.

15. The method of claim 14 in which either the 1-2 bond or the 3-4 bond is $-(CH_2-NH)-$.

16. The method of claim 10 in which the compound is selected from the group consisting of:

A. Divalinal angiotensin IV [^+H_3N-Val(CH_2NH)Tyr-Val(CH_2NH)-His-Pro-Phe-COO^-];

B. D-Val_1-angiotensin IV (i.e., angiotensin IV with a D-valine residue in the 1 position);

C. Norleucine-TYR-ILE-GLY-GLY-DPHE; and

D. Norleucine-TYR-ILE-HN-$(CH_2)_6$-NH_2.

17. The method of claim 11 in which the antagonist has formula D.

18. The method of claim 2, wherein the compound inhibits aminopeptidase A.

19. The method of claim 2 wherein the compound inhibits aminopeptidase M.

20. The method of claim 2 wherein the compound comprises amastatin.

21. A therapeutic composition comprising a therapeutically effective amount of an angiotensin IV antagonist of Formula I, II, III, IV, or V.

22. The therapeutic composition of claim 21, wherein the angiotensin IV antagonist is a compound having the following general formula, or an acetate salt thereof:

A - B - C - HIS - PRO - D

where A = SAR or VAL
B = TYR OR TRP
C = ILE or VAL and
D = ALA or PHE;

provided that if A is VAL and B is TYR and C is ILE, then D is ALA.

23. The therapeutic composition of claim 22 wherein the antagonist of angiotensin IV is selected from the group consisting of the following compounds and acetate salts thereof:

VAL-TYR-ILE-HIS-PRO-ALA;
VAL-TRP-ILE-HIS-PRO-ALA;
VAL-TRP-VAL-HIS-PRO-ALA;

24. A therapeutic composition comprising an antibody specific for angiotensin IV or an Fab fragment derived from such an antibody.

25. A method of screening a compound for the ability to promote fibrinolysis, comprising

providing a mixture comprising angiotensin IV or an agonist of angiotensin IV, a receptor specific for angiotensin IV, and the compound, and

determining whether the compound inhibits binding of angiotensin IV to the receptor.

26. A method of screening a compound for the ability to promote fibrinolysis, comprising

providing a precursor to angiotensin IV selected from angiotensin II and angiotensin III,

providing one or more aminopeptidases capable of converting the precursor to a product, and

determining the ability of the compound to inhibit formation of the product.

27. A method of inhibiting fibrinolysis in a patient by providing a therapeutically effective amount of angiotensin IV or an agonist thereof.

28. The method of claim 27, wherein the patient has a disorder characterized by inadequate clotting.

29. The method of claim 27 wherein the patient has a condition selected from the group consisting of afibrinogenemia, dysfibrinogenemia, hypoprothrombinemia, parahemophilia, hypoconvertinemia, hemophilia A, hemophilia B, Stuart-Prower factor deficiency, plasma thromboplastin antecedent deficiency, Hageman trait, thrombocytopenia, disorders of platelet function, Von Willebrand's disease, hepatic dysfunction, circulating anticoagulants, inherited defects in natural coagulation inhibitors, dysplasminogenemia, defective release or diminished venous content of plasminogen activator, excessive release of PAI, heparin cofactor II deficiency, homocystinuria, chronic congestive heart failure,

metastatic tumor or malignancy, extensive trauma or major surgery, myeloproliferative disorders, and treatment with oral contraceptives or L-asparaginase.

30. An antibody specific for angiotensin IV.

31. A physiologically acceptable composition comprising a therapeutic amount of angiotensin IV.

32. A physiologically acceptable composition comprising a therapeutic amount of an inhibitor of aminopeptidase A or aminopeptidase M.

ANGIOTENSIN DEGRADATION SEQUENCE

PEPTIDE	SEQUENCE	INHIBITORS
Angiotensin I	D-R-V-Y-I-H-P-F-H-L	
◄ ◄ ◄ ACE		Captopril, etc.
Angiotensin II	D-R-V-Y-I-H-P-F	
◄ ◄ ◄ Aminopeptidase A		Amastatin, EDTA
Angiotensin III	R-V-Y-I-H-P-F	
◄ ◄ ◄ Aminopeptidase M		Amastatin
Angiotensin IV	V-Y-I-H-P-F	

FIG. 1

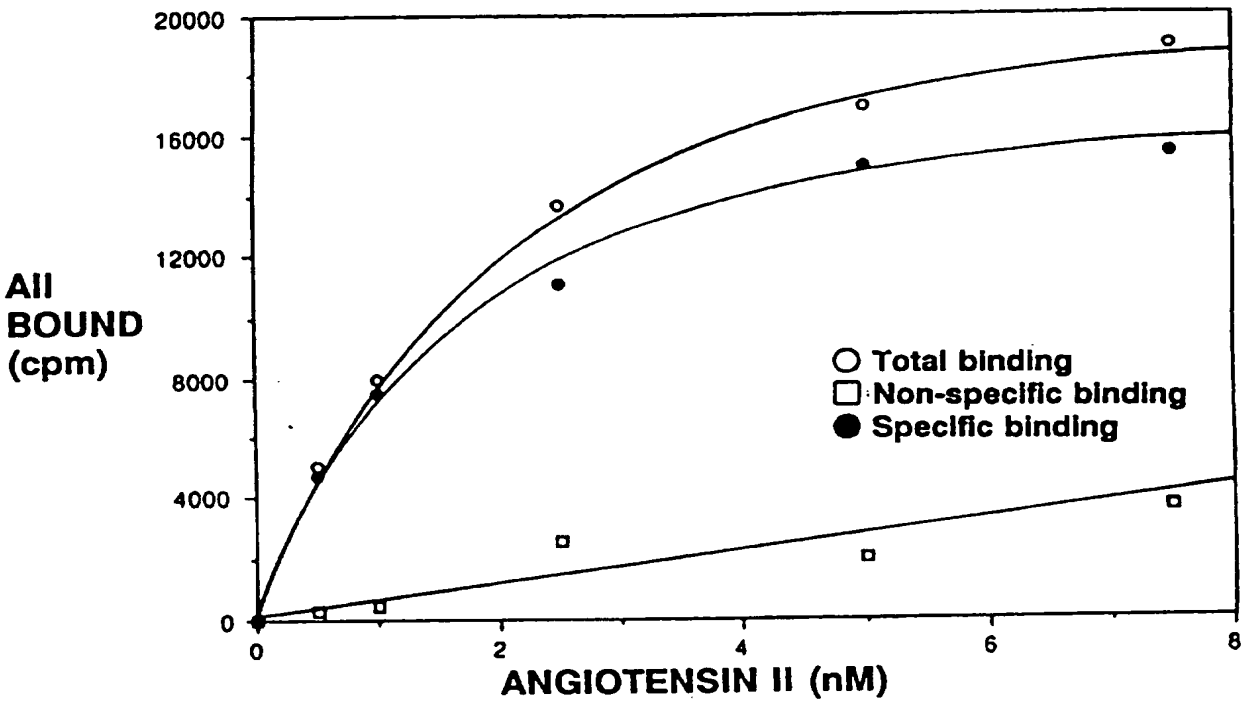
20000
16000
12000
8000
4000
0
AII BOUND (cpm)
0
2
4
6
8
ANGIOTENSIN II (nM)
Total binding
Non-specific binding
Specific binding

FIG. 2

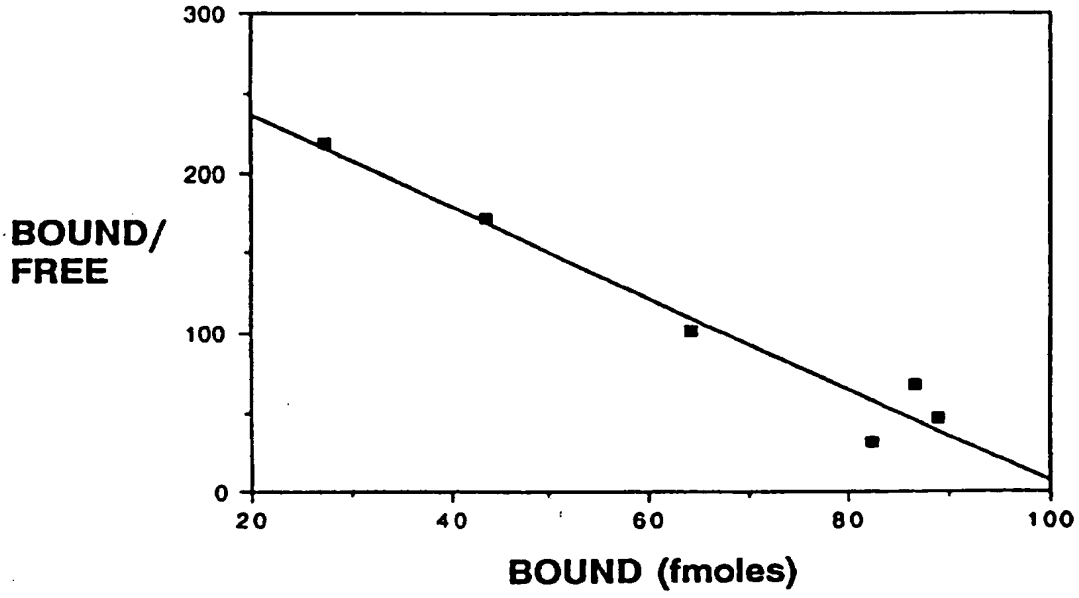
300
200
100
0
BOUND/ FREE
20
40
60
80
100
BOUND (fmoles)

FIG. 3

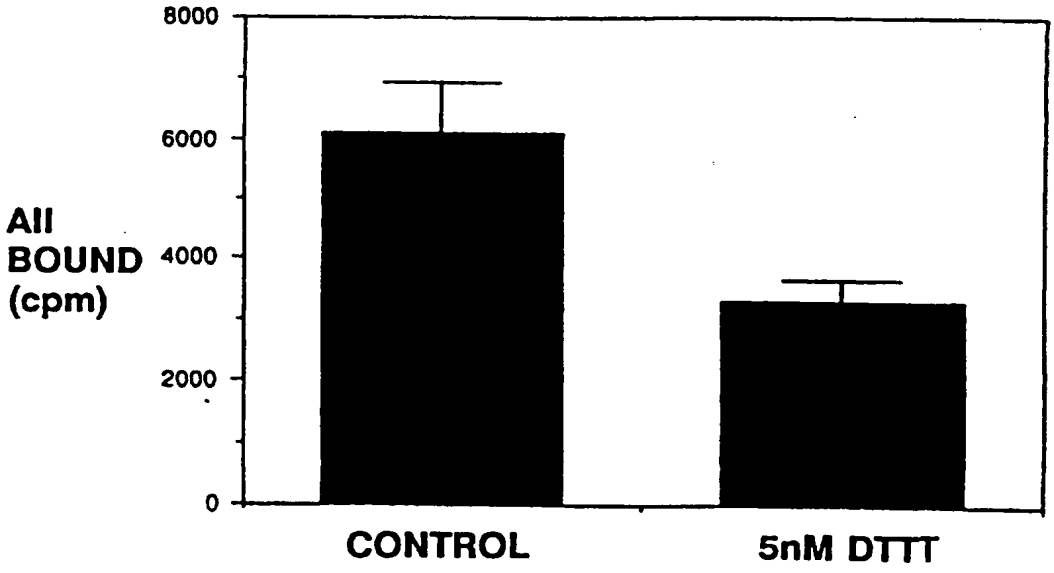
8000
6000
4000
2000
0
AII BOUND (cpm)
CONTROL
5nM DTTT

FIG. 4

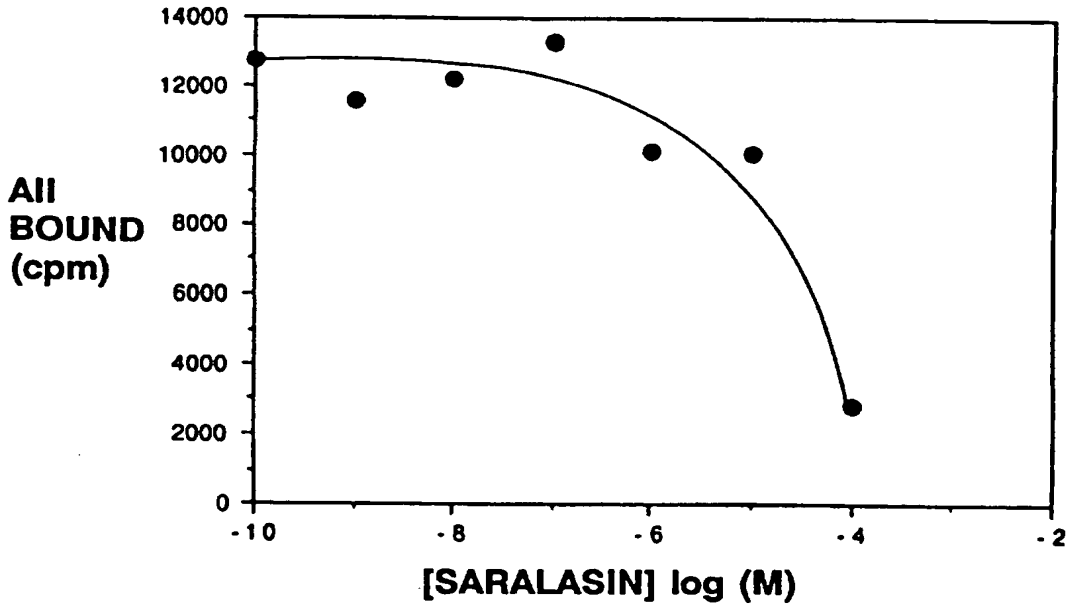
14000
12000
10000
8000
6000
4000
2000
0
AII BOUND (cpm)
-10
-8
-6
-4
-2
[SARALASIN] log (M)

FIG. 5

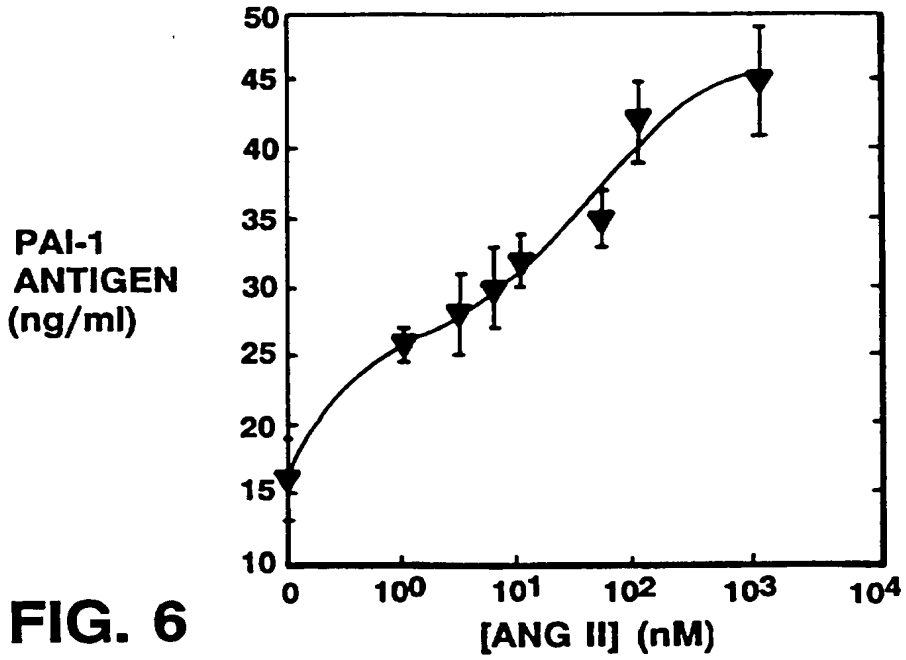
PAI-1
ANTIGEN
(ng/ml)
50
45
40
35
30
25
20
15
10
0
10^0
10^1
10^2
10^3
10^4
[ANG II] (nM)

FIG. 6

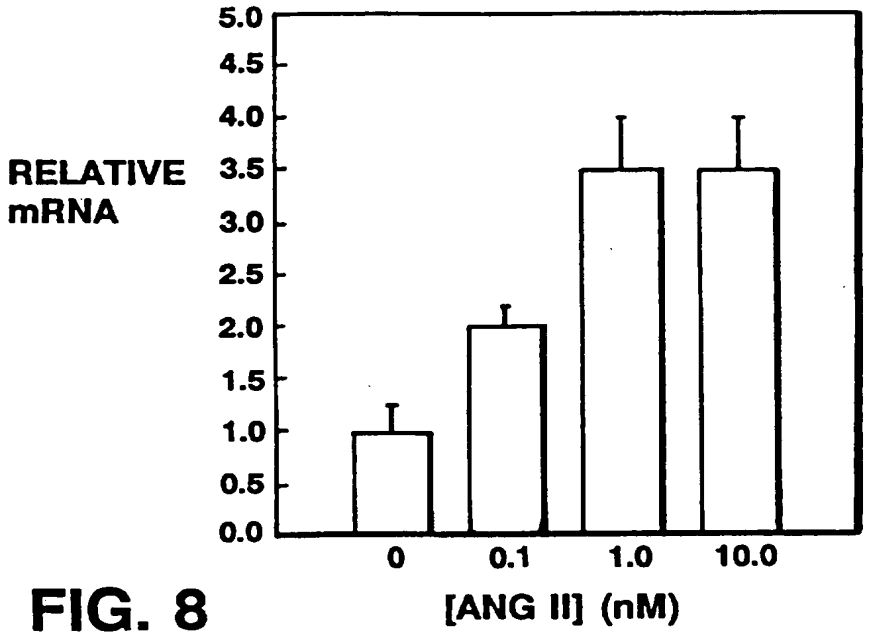
RELATIVE
mRNA
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
0.1
1.0
10.0
[ANG II] (nM)

FIG. 8

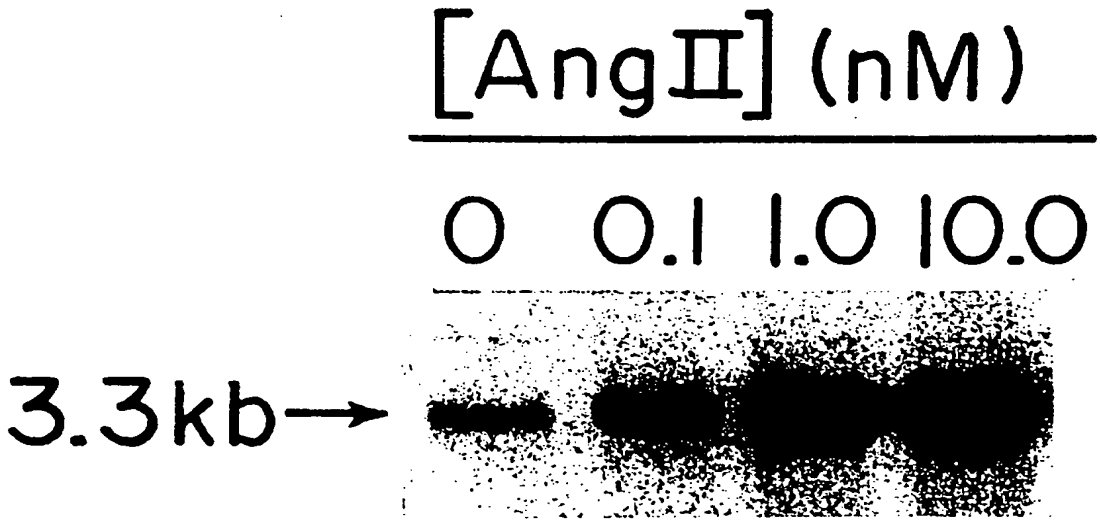

FIG. 7

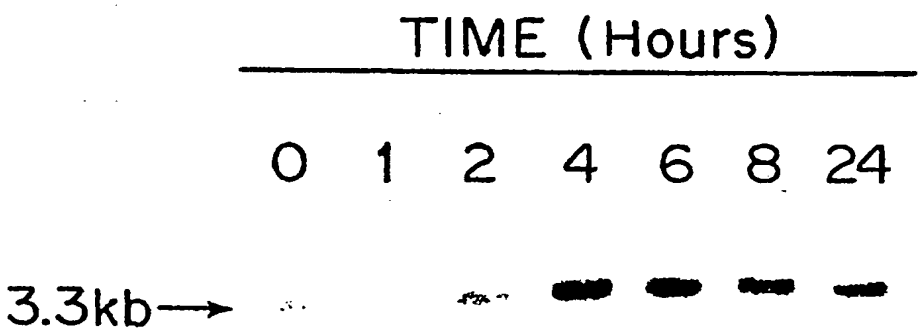

FIG. 9

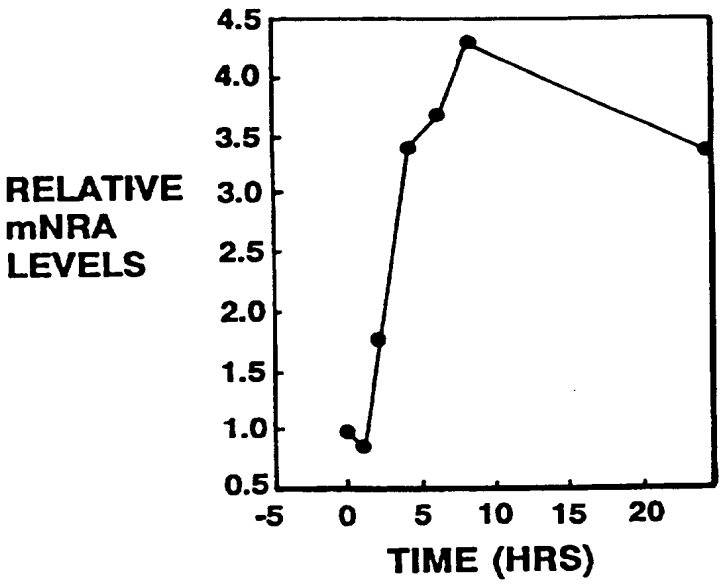

FIG. 10

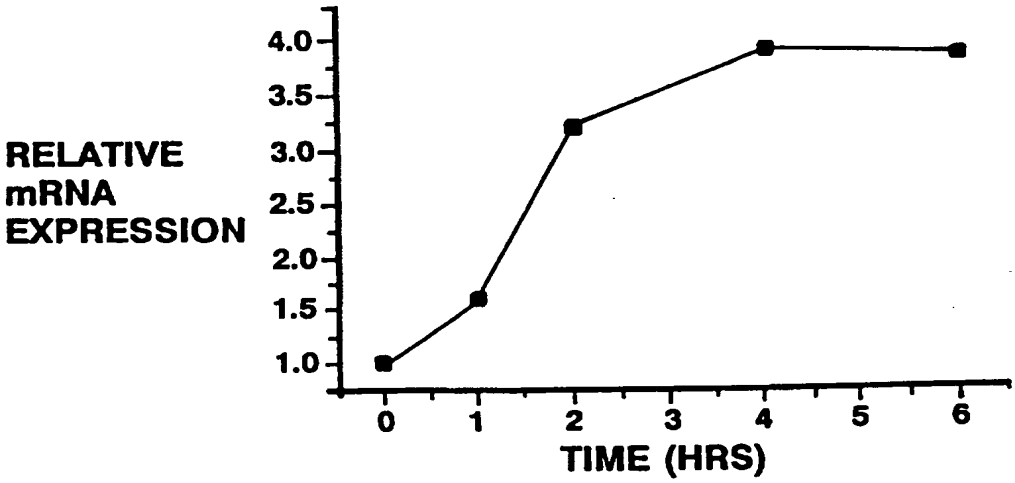

FIG. 15B

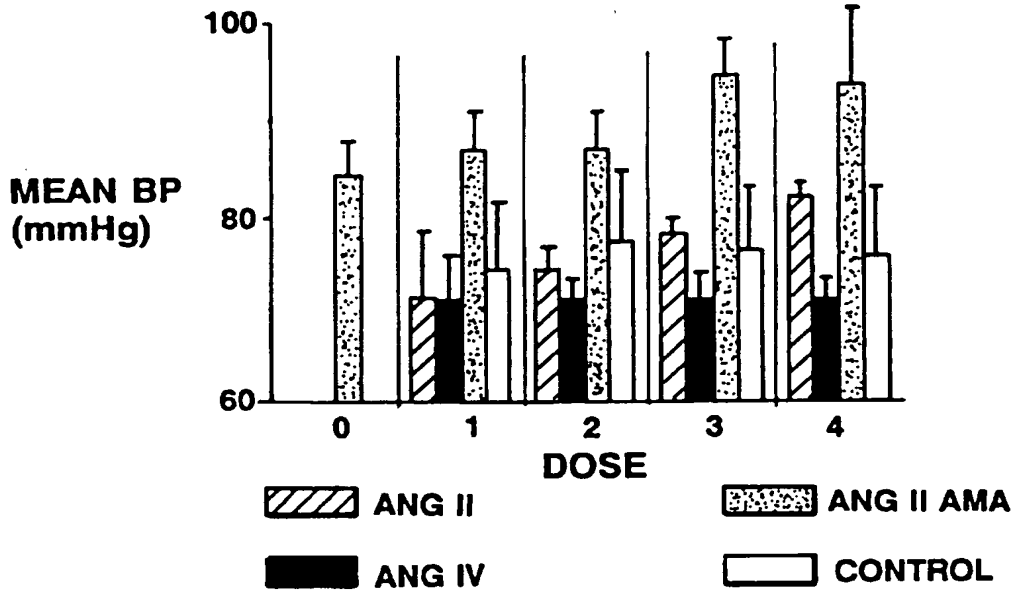
MEAN BP
(mmHg)
100
80
60
0
1
2
3
4
DOSE
ANG II
ANG II AMA
ANG IV
CONTROL

FIG. 11

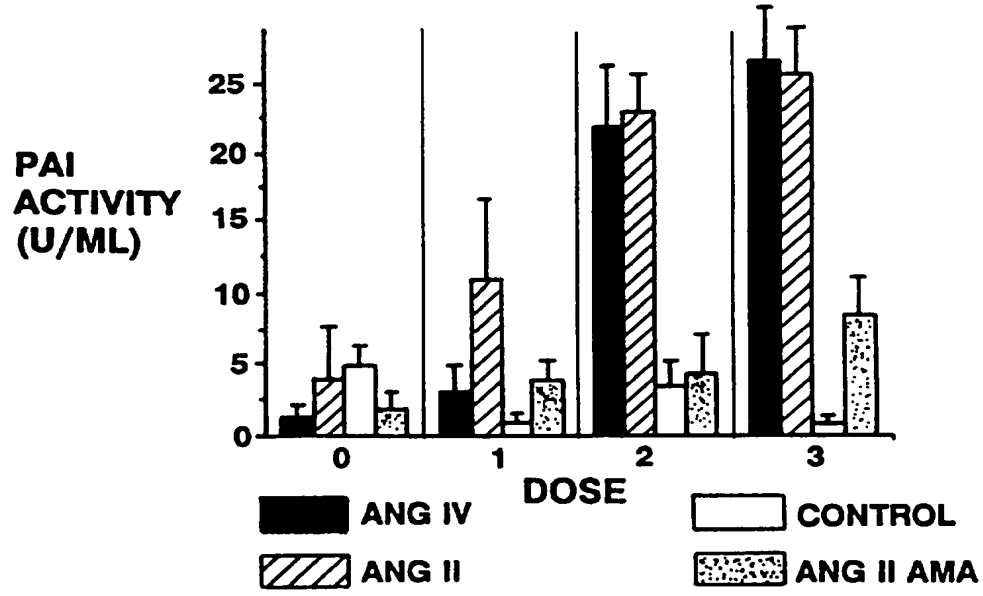
PAI
ACTIVITY
(U/ML)
25
20
15
10
5
0
0
1
2
3
DOSE
ANG IV
CONTROL
ANG II
ANG II AMA

FIG. 12

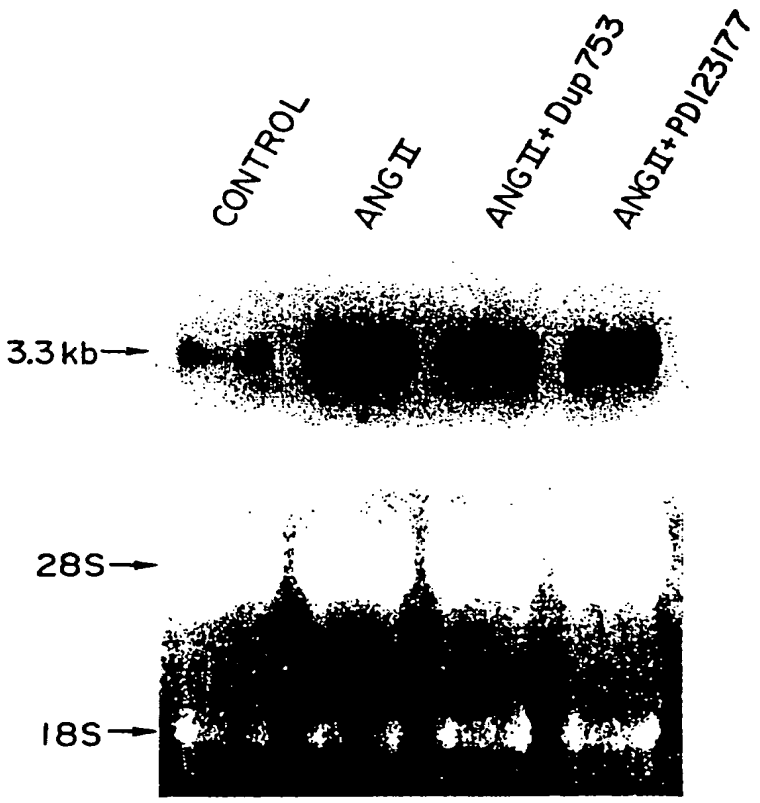
CONTROL
ANG II
ANG II + Dup 753
ANG II + PD123177
3.3 kb
28S
18S

FIG. 13

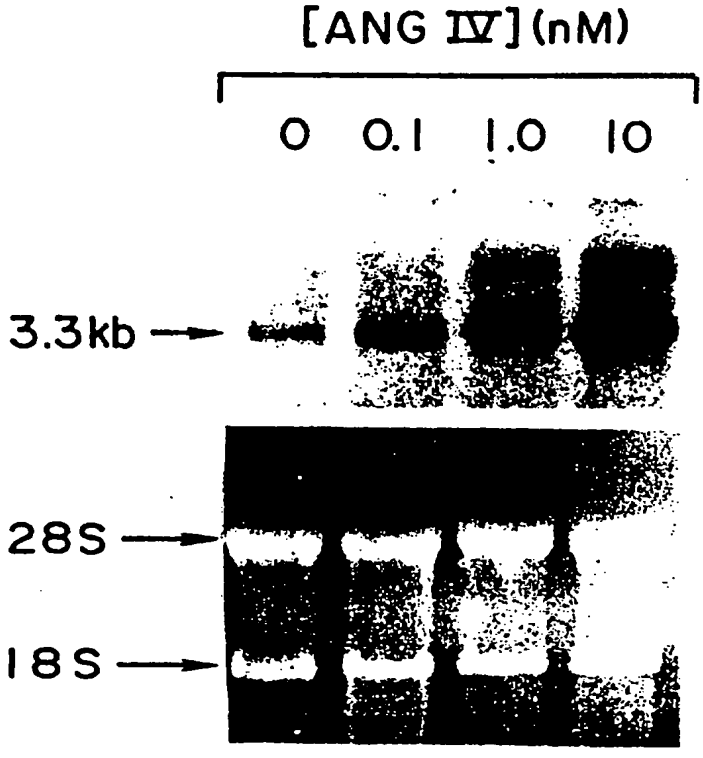
[ANG IV] (nM)
0
0.1
1.0
10
3.3kb
28S
18S

FIG. 14

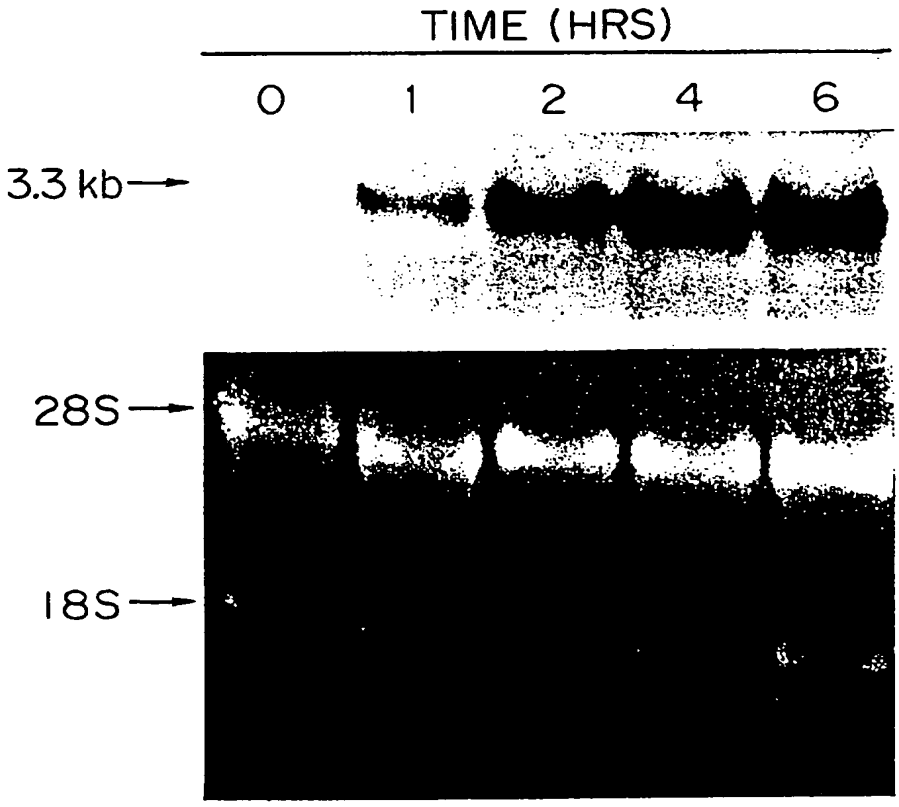

FIG. 15A

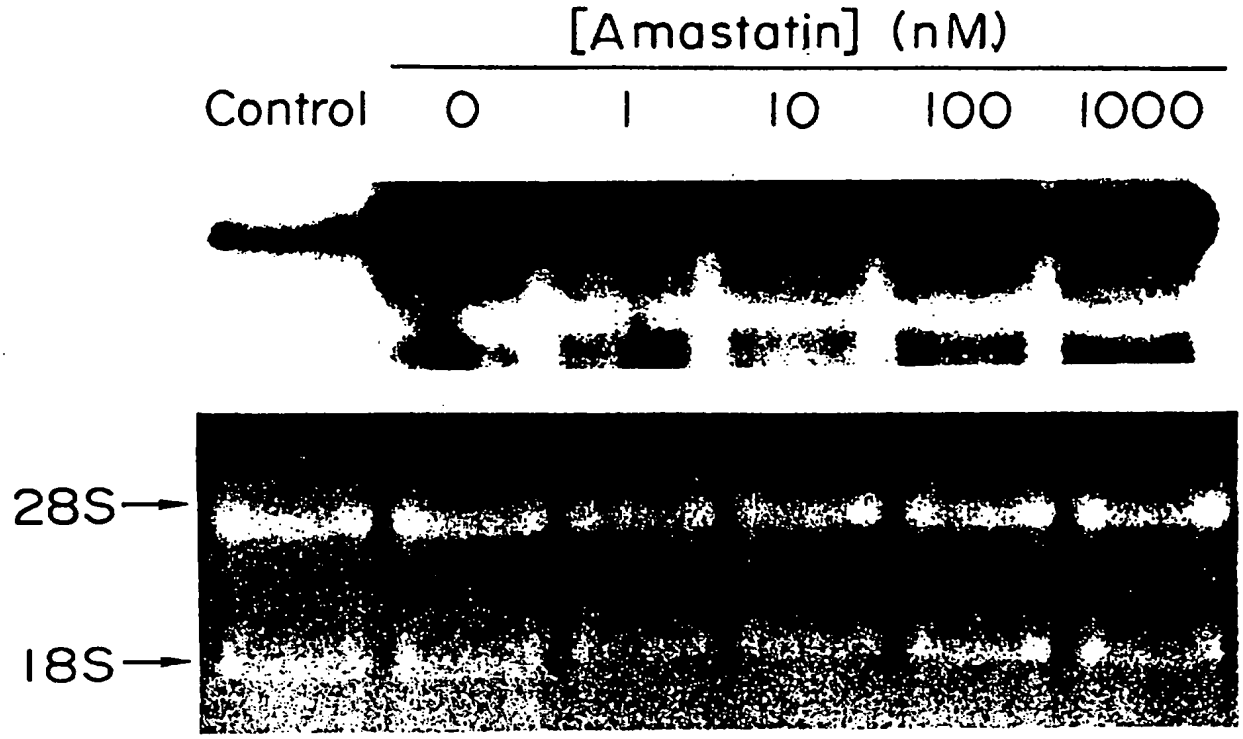

FIG. 16

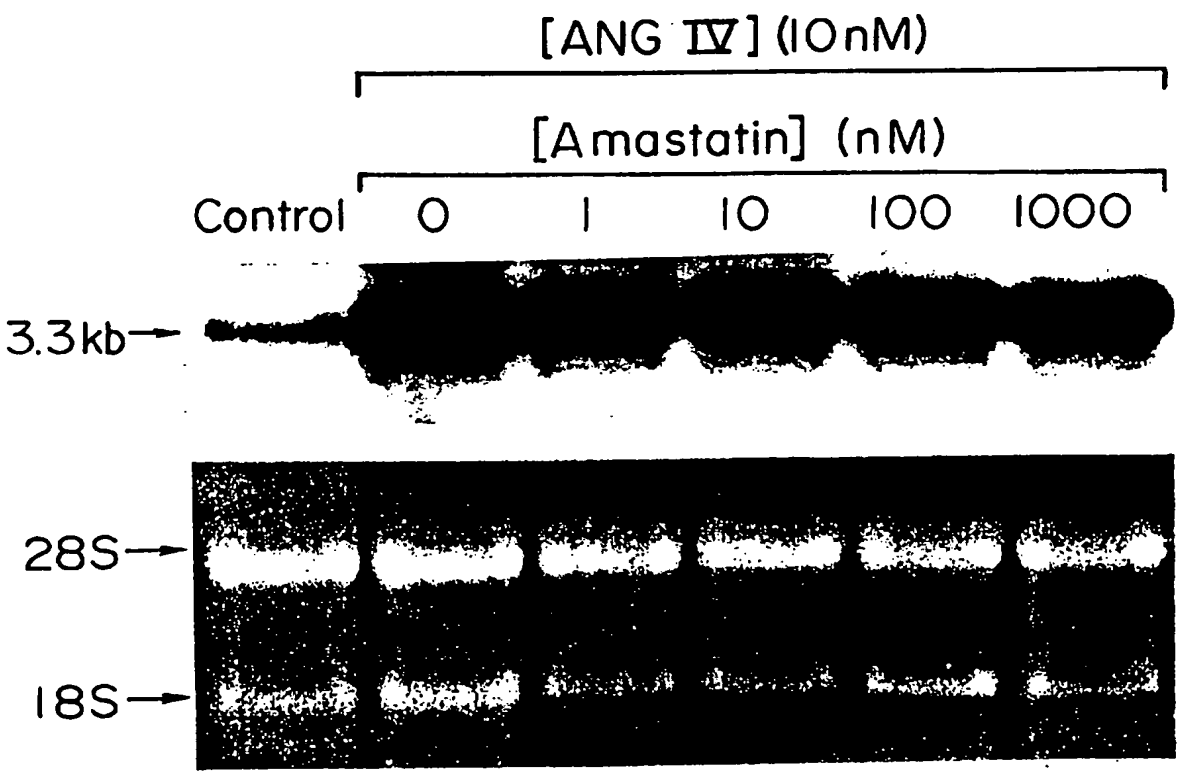
[ANG IV] (10nM)
[Amastatin] (nM)
Control
0
1
10
100
1000
3.3kb
28S
18S

FIG. 17

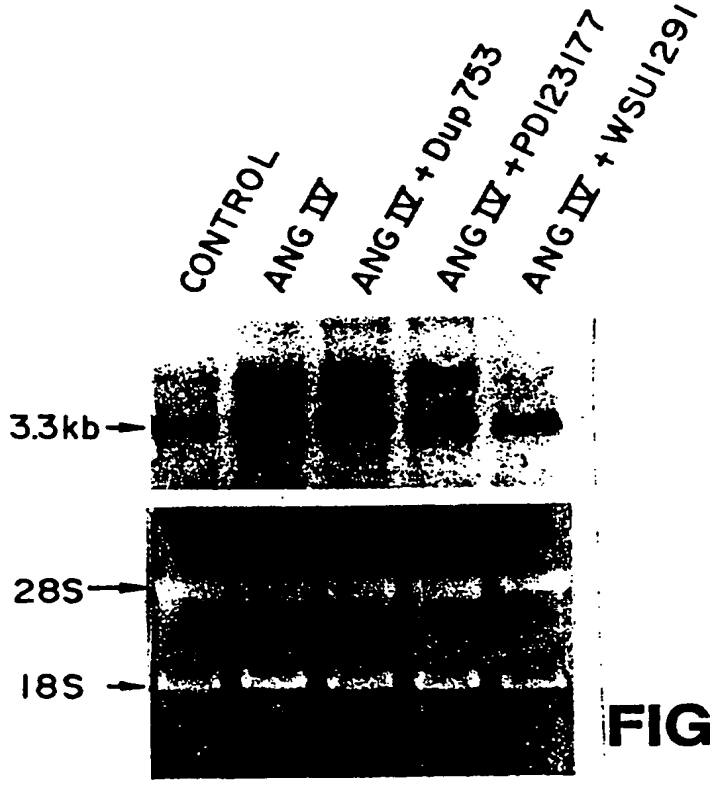
CONTROL
ANG IV
ANG IV + Dup 753
ANG IV + PD123177
ANG IV + WSU1291
3.3kb
28S
18S

FIG. 18

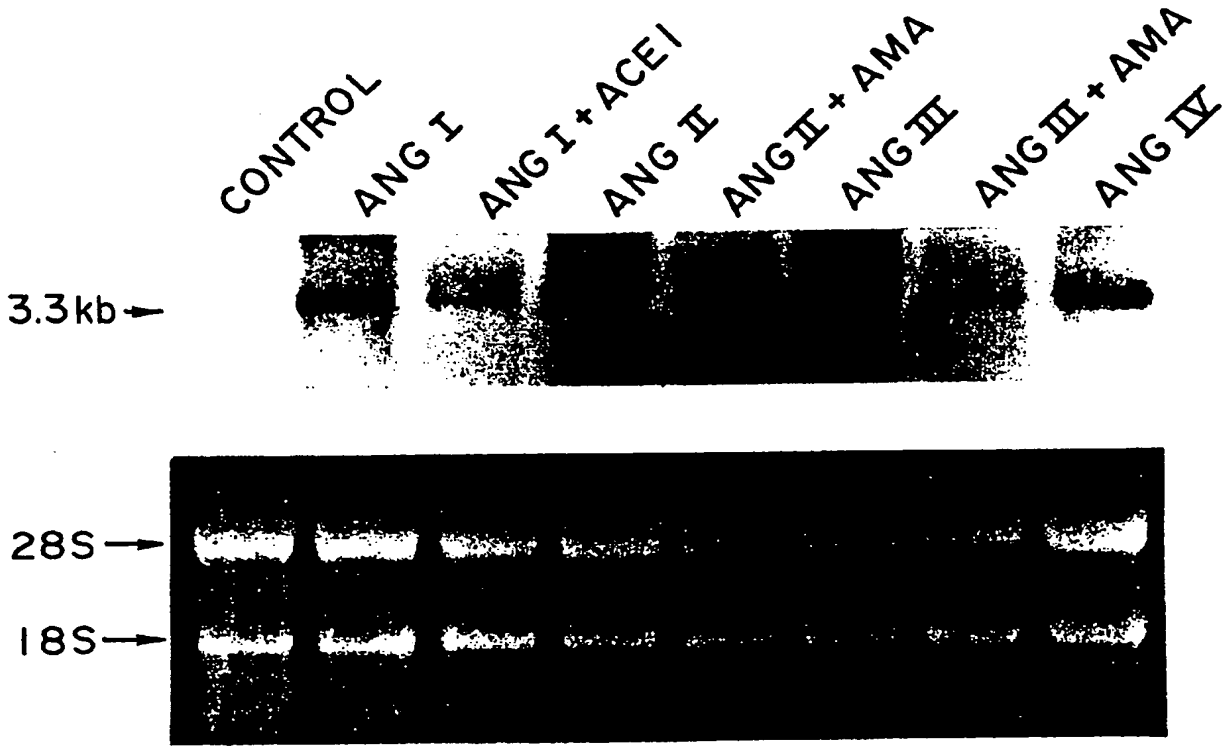
CONTROL
ANG I
ANG I + ACE I
ANG II
ANG II + AMA
ANG III
ANG III + AMA
ANG IV
3.3kb
28S
18S

FIG. 19

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/13804

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 38/00, 38/04; A01N 37/18; C07K 7/06, 7/14
US CL :514/1, 2; 530/300, 329
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/1, 2; 530/300, 329

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DIALOG, MEDLINE, CAB ABSTRACTS, DERWENT WPI, DERWENT BIOTECHNOLOGY ABSTRACTS, JAPIO, EMBASE, BIOSYS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	NAVERI et al. Angiotensin IV reverses the acute cerebral blood flow reduction after experimental subarachnoid hemorrhage in the rat. Journal of Cerebral Blood Flow and Metabolism. 1994, Vol. 14, No. 6, pages 1096-1099, see entire document.	1, 3-8, 22 and 23
A	NAVERI, L. et al. The role of angiotensin receptor subtypes in cerebrovascular regulation in the rat. Acta Physiologica Scandinavica. 1995, Vol. 155, Supplementum 630, pages 1-48, see entire document.	1, 3-8, 22 and 23

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 01 NOVEMBER 1996	Date of mailing of the international search report 26 NOV 1996
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer PATRICIA A. DUFFY Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/13804

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	SARDINIA et al. AT_4 receptor structure-binding relationship: *N*-terminal-modified angiotensin IV analogues. Peptides. 1994, Vol. 15, No. 8, pages 1399-1406, see entire document.	1, 3-8, 22 and 23
A	WRIGHT et al. The angiotensin IV system: functional implications. Frontiers in Neuroendocrinology. January 1995, Vol. 16, pages 23-52, see entire document.	1, 3-8, 22 and 23
A	WO 94/00492 A1 (WASHINGTON STATE UNIVERSITY RESEARCH FOUNDATION) 01 June 1994 (01.06.94), see entire document.	1, 3-8, 22 and 23

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/13804

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1, 3-8, 22 and 23

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/13804

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1.

Group I, claims 1, 3-17, 21-24, and 30, drawn to methods of promoting fibrinolysis by administering a therapeutic amount of an antagonist of angiotensin IV.

Group II, claims 2-6, 18-20 and 32, drawn to methods of promoting fibrinolysis by administering a therapeutic amount of an a compound which inhibits the conversion of angiotensin II to angiotensin IV.

Group III, claim 25, drawn to a method of screening for the ability of a compound to promote fibrinolysis by determining the ability of the compound to inhibit binding of angiotensin IV to its receptor.

Group IV, claim 26, drawn to a method of screening for the ability of a compound to promote fibrinolysis by determining the ability of the compound to inhibit the formation of angiotensin IV.

Group V, claims 27-29, drawn to method of inhibiting of fibrinolysis by administering angiotensin IV or agonist thereof.

Group VI, claim 31, drawn to a therapeutic compound comprising angiotensin IV.

The special technical features of each of the Groups I-VI are as follows:
Group I - the antagonists of angiotensin IV.
Group II - compounds which inhibit the conversion of angiotensin II to angiotensin IV.
Group III - angiotensin IV receptor.
Group IV - aminopeptidases.
Group V - method of inhibiting fibrinolysis.
Group VI - angiotensin IV.

and it considers that the International Application does not comply with the requirements of unity of invention (Rules 13.1, 13.2 and 13.3) for the reasons indicated below:

Group I does not share a special technical feature with each of Groups II-VI because they fail to share the same special technical feature from both a chemical and biological perspective. The special technical features of Groups I-VI fail to share a similar chemical core structure. Moreover, the special technical features all have different biological functions. For, example the compounds of Group II inhibit the enzymatic conversion of angiotensin II to angiotensin IV, while others are enzymes themselves (i.e. aminopeptidases of Group IV). The method and agonists of Group V and VI are distinct from Group I in that the compounds have different biological functions (i.e. inhibit fibrinolysis) versus Group I (promote fibrinolysis). As a result, the claims are not so linked by a special technical feature within the meaning of PCT Rule 13.2 so as to form a single inventive concept.

This application contains claims directed to more than one species of the generic invention. These species are deemed to lack Unity of Invention because they are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for more than one species to be examined, the appropriate additional examination fees must be paid. The species are as follows:
Group I, claims 1, 3-17, 21-24 and 30, drawn to methods of promoting fibrinolysis by administering a therapeutic amount of an antagonist of angiotensin IV.
Species A - the peptides having the common sequence A-B-C-HIS-PRO-D (claims 1, 3-8, 22 and 23).
Species B - an antibody which specifically binds angiotensin IV (claims 9, 24 and 30).
Species C - the peptide analogs of angiotensin IV, which inhibit binding of angiotensin IV to its receptor (claims 10-17 and 21).

The peptide species (A and C) do not share a special technical feature because they fail to share a common core structure. The peptides of Species C fail to share the common core structure of HIS-PRO as set forth for the peptides of Species A. Moreover, the peptides of Species C function to inhibit binding of angiotensin IV to its receptor which is not required by Species A. Species B (an antibody which bind angiotensin IV) does not share a special technical feature with each of A and C because they fail to have a common structure and have different biological properties. In the instant case the antibody of Specie B specifically binds angiotensin whereas species A and C do not. Specie B does not share a special technical feature with Species C which are drawn to angiotensin IV receptor antagonists because

species B does not bind the receptor per se but binds the ligand for the receptor. Angiotensin IV, peptide antagonists are known in the art and thus do not provide an inventive contribution over the prior art as a whole and are not so structurally or chemically linked so as to form a single inventive concept under PCT Rule 13.1.

AN - 97-271879 [24]
AP - WO96US13804 960827 AU960068617 960827; [Based on WO9716201]
PR - US950550174 951030
- Use of angiotensin IV antagonists or agonists - for promoting or inhibiting fibrinolysis in patients for treating conditions such as blood clotting disorders
IW - ANGIOTENSIN IV ANTAGONIST AGONIST PROMOTE INHIBIT FIBRINOLYTIC PATIENT TREAT CONDITION BLOOD CLOT DISORDER
PA - (BGHM) BRIGHAM & WOMENS HOSPITAL
- (UNIW) UNIV WASHINGTON STATE RES FOUND
PN - WO9716201 A1 970509 DW9724 A61K38/00 Eng 066pp
- AU6861796 A 970522 DW9739 A61K38/00 000pp
IC - A01N37/18 ; A61K38/00 ; A61K38/04 ; C07K7/06 ; C07K7/14

Continue: Y / N
? y
CT - 4.Jnl.Ref; WO9400492
AB - WO9716201 A method of promoting fibrinolysis in a patient is claimed, comprising administering an antagonist of angiotensin IV or a compound which inhibits the conversion of angiotensin II to angiotensin IV. The antagonist is e.g. an antibody specific for angiotensin IV or a compound of formula A-B-C-His-Pro-D (I), where A = Sar or Val; B = Tyr or Trp; C = Ile or Val; and D = Ala or Phe; provided that if A = Val and B = Tyr and C = Ile, then D = Ala. Also claimed is a method of inhibiting fibrinolysis in a patient, comprising providing angiotensin IV or an agonist of angiotensin IV.
- USE - The promotion of fibrinolysis can be used for e.g. treating thromboembolic disorders, congestive heart failure, cardiomyopathy, myocardial infarction or cerebrovascular disease, for prophylaxis of

Continue: Y / N
? y
undesired clotting as a result of surgery, and for post-surgical maintenance of grafts or prostheses (all claimed). The inhibition of fibrinolysis can be used for treating a patient with e.g. afibrinogenaemia, dysfibrinogenaemia, hypothrombinaemia, parahaemophilia, hypoconvertinaemia, haemophilia A and B, Stuart-Prower factor deficiency, plasma thromboplastin antecedent deficiency, Hageman trait, thrombocytopenia, disorders of platelet function, Von Willebrand's disease, hepatic dysfunction, circulating anticoagulants, inherited defects in natural coagulation inhibitors, dysplasminogenaemia, defective release or diminished venous content of plasminogen activator, excessive release of plasminogen activator inhibitor (PAI), heparin cofactor II deficiency, homocystinuria, chronic congestive heart failure, metastatic tumour or malignancy, extensive trauma or major surgery, myeloproliferative disorders, or

Continue: Y / N
? y
treatment with oral contraceptives or L-asparaginase (all claimed).

D5